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Teratogenicity of Cyclophosphamide in a Coupled Microsomal Activating/Embryo Culture System

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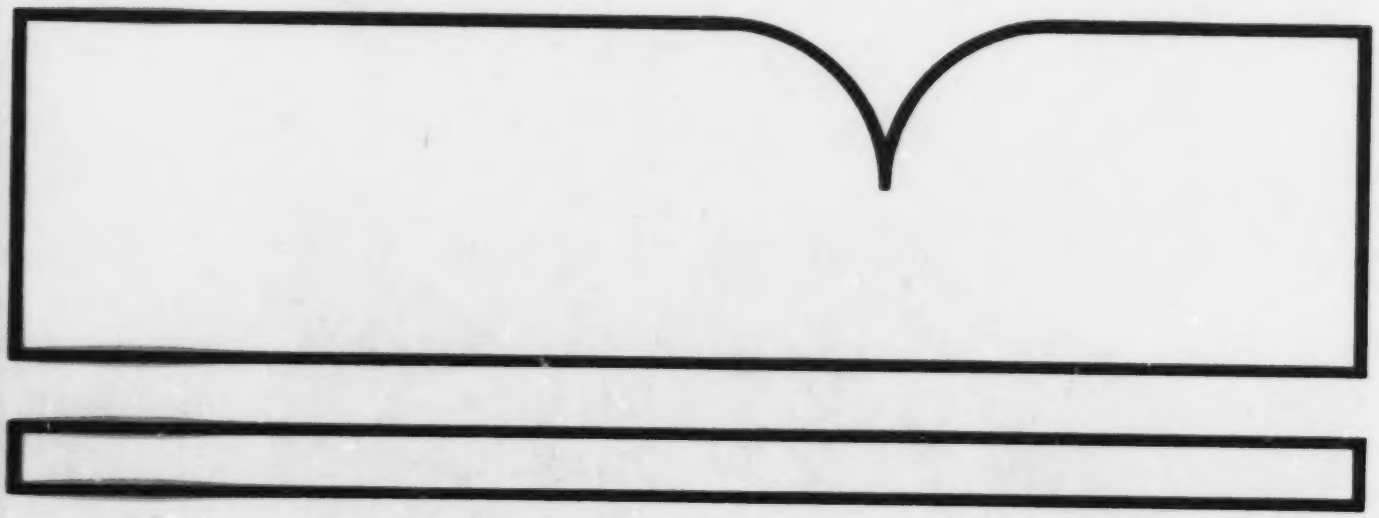

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16. ABSTRACT

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TERATOGENICITY OF CYCLOPHOSPHAMIDE IN A COUPLED MICROSOMAL ACTIVATING/EMBRYO CULTURE SYSTEM*

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Abstract—Using the coupled microsomal activating/embryo culture system, *in vitro* experiments were performed to establish the role of metabolism in the embryo toxicity and teratogenicity of cyclophosphamide. Cyclophosphamide in the coupled microsomal activating/embryo culture system produced characteristic morphological lesions as well as a general inhibition of embryo and yolk sac growth. Increasing concentrations of NADPH in the presence of microsomes and cyclophosphamide produced progressively greater responses. These effects did not occur when microsomes and NADPH were present in the serum medium for the first 2 hours of incubation followed by one washing and then culturing of the conceptuses from hr 2 to hr 48 in a medium containing cyclophosphamide alone. Cytochrome P-450-depleted microsomes did not bioactivate cyclophosphamide to teratogenic or toxic metabolites. The results indicate that cytochrome P-450-dependent microsomal metabolism of cyclophosphamide is required for the embryotoxic and teratogenic effects observed *in vitro*.

The importance of metabolism of chemicals in carcinogenesis and mutagenesis is well known. In contrast, the role of maternal metabolism in teratology and reproductive toxicology remains a disputed point. The embryo and fetus are usually considered to have little or no biotransforming ability. Moreover, the developing embryo and fetus are protected from maternally generated metabolites by the placenta and yolk sac barriers as well as by the long distances and times required for transit of activated unstable metabolites to reach the conceptus.

Cyclophosphamide, a widely used chemotherapeutic agent, induces developmental anomalies in offspring of laboratory animals *in vivo* [1–5]. Cyclophosphamide is transformed into active components by the mixed-function oxidase enzyme system [6] located in the microsomes of the liver; these active metabolites cause retardation of neoplastic cell multiplication in culture [6]. Although the molecular mechanisms of cyclophosphamide-induced teratogenesis and reproductive toxicity remain unknown, it is possible that a process of maternal bioactivation may produce teratogenetic metabolites affecting embryonic growth and development. *In vivo* teratogenesis studies have shown, however, that phenobarbital pretreatment reduces, and SKF-525A pretreatment increases, the teratogenic effect of cyclophosphamide [5].

In contrast, other investigators have provided *in vitro* evidence that cyclophosphamide may have to be metabolized to be embryotoxic or teratogenic. For example, 4-ketocyclophosphamide produces defects in cultured limb buds [7]. Hamster embryo cells (a biotransformation source) causes increased incorporation of label from [^{14}C]cyclophosphamide into mouse limb buds grown in culture [8]. Other investigators have added a post-mitochondrial supernatant fraction obtained from Aroclor 1254-pretreated rats to human serum used to culture rat embryos [9]. These investigators concluded that maternal drug metabolism is a major determinant of cyclophosphamide-induced embryo teratogenicity.

In the study presented here, the role of cytochrome P-450-dependent microsomal metabolism in an *in vitro* embryo culture system has been examined. The effect of increasing concentrations of NADPH on the coupled microsomal activating/embryo culture system was used to differentiate non-cytochrome P-450 microsomal metabolism of cyclophosphamide from cytochrome P-450-dependent microsomal metabolism. Embryos were also cultured in medium containing microsomes and 1.0 mM NADPH for 2 hr, washed, and then put in fresh medium containing cyclophosphamide, to determine the relative importance of metabolism compared to possible mechanisms involving a damaged yolk sac. Cumene hydroperoxide-pretreated, and thus cytochrome P-450-depleted, microsomes were also used in the coupled microsomal activating/embryo culture system

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to further define the role of cytochrome P-450-dependent metabolism in the *in vitro* embryo toxicity and teratogenicity of cyclophosphamide.

METHODS

Liver microsomes. Hepatic microsomes from adult male rats were obtained as described previously [10]. To inactivate cytochrome P-450, some hepatic microsomes were suspended in 0.05 M Tris–HCl, pH 7.5, containing 0.15 M KCl at a concentration of 3–4 mg protein/ml, and were incubated with 2.0 mM cumene hydroperoxide for 20 min at 37°. Glutathione (5.0 mM) was then added and the incubation was continued for an additional 3 min. The microsomes were cooled for 10 min in ice water and spun at 105,000 *g* for 60 min. The pellet was resuspended and spun for a third time and, finally, suspended in cold rat serum before use in culturing experiments. Using absorption spectroscopy as the criterion, no residual cumene hydroperoxide was found in the treated microsomes after glutathione treatment. The ability of microsomes to *N*-demethylate aminopyrine to formaldehyde [11] was determined in 0.05 M Tris–HCl buffer, pH 7.5, or in rat serum gassed with 20% O_2, 5% CO_2, and 75% N_2.

The aminopyrine *N*-demethylase activations were determined by the method of Lucier *et al.* [12], using 1.8 mM aminopyrine with an incubation time of 15 min. Cytochrome P-450 determinations were performed by the method of Omura and Sato [13].

Conceptus culture and treatment groups. Rat conceptuses of pregnancy day 11 (sperm positive is day 1) were cultured for 48 hr in rotating 30 ml serum bottles according to methods described earlier [14–16]. They were grown in fresh serum obtained from 75- to 80-day-old male rats. The incubation serum contained 50 μg/ml streptomycin and 50 I.U./ml penicillin (GIBCO, Grand Island, NY). Each conceptus was cultured in 5 ml fresh serum medium with a 20% O_2, 5% CO_2, and 75% N_2 gas atmosphere for 20 hr and with 40% O_2, 5% CO_2, and 55% N_2 for the next 28 hr [17]. During the afternoons (3:00 to 5:00 p.m.), conceptuses, with embryos rotated into concave fetal position (Witschi state 16), were collected from several rats and were distributed randomly to the various treatment groups. In some experiments, conceptuses were cultured for 2 hr, removed from the original incubation media, washed three times in Trowell's T8 tissue culture medium (GIBCO) for a total of 20 min, put in fresh serum, and regassed with 20% O_2, 5% CO_2, and 75% N_2, before the culturing continued. Cyclophosphamide was included in the incubation media at a level of 100 μg/ml (0.35 mM) where indicated. NADPH concentration was 1.0 mM unless otherwise indicated. A concentration of rat hepatic microsomes between 0.4 and 0.5 mg protein/ml was normally used.

At the end of the 48 hr culture period, conceptuses were removed from the medium and washed in 5 ml of Ringer's solution. The yolk sac diameter, beating of the heart, and somite number were recorded using a binocular stereomicroscope. On the basis of gross microscopic observation, embryos were classified as either normal or abnormal when compared to untreated embryos cultured *in vitro*. The term "abnormal embryo" includes both severe growth retardation that prevents normal *in vitro* differentiation as well as specific dysmorphogenesis. The embryo and yolk sac dissected from each conceptus was frozen in liquid nitrogen and stored at −15° until used (within 2–3 weeks) for DNA and protein measurements. These tissues were homogenized in 1 ml of ice-cold 0.05 M phosphate buffer, pH 7.4, with a sonifier (Branson Sonic Power Co.). DNA was estimated by the ethidium bromide fluorescence method after digestion of aliquots of homogenate with RNase and protease [18]. Protein was measured using the method of Lowry *et al.* [19].

Chemicals and statistics. NADPH was purchased from the Sigma Chemical Co. (St. Louis, MO). Cyclophosphamide was obtained from Mead & Johnson, Inc. (Evansville, IN). Cumene hydroperoxide and reduced glutathione were purchased from Matheson, Coleman & Bell (Norwood, OH) and CalBiochem (La Jolla, CA) respectively. The Aldrich Chemical Co. (Milwaukee, WI) supplied the aminopyrine.

Quantitative data are represented as mean ± S.E.M. (N) for each treatment group; significance was evaluated using analysis of variance and Student's *t*-test [20]. Enumerative data are expressed as the incidence of occurrence; the statistical significance was calculated using Chi-square contingency tables (exact method) [21].

RESULTS

The data presented in Fig. 1 show the effects of cyclophosphamide on embryonic and yolk sac growth. Cyclophosphamide (0.35 mM, 100 μg/ml) *per se* did not inhibit embryonic or yolk sac growth or cause abnormal differentiation of the embryo. In the coupled microsomal activating/embryo culture

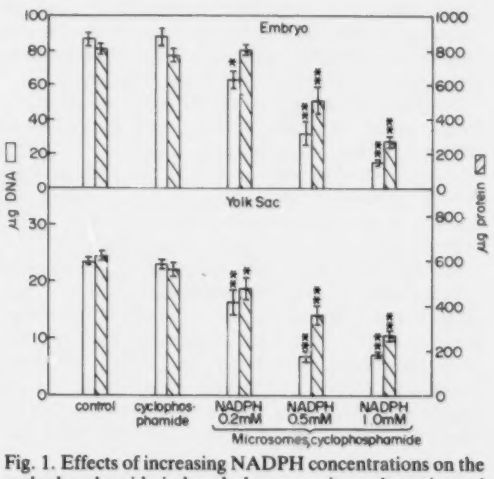

Fig. 1. Effects of increasing NADPH concentrations on the cyclophosphamide-induced decreases in embryonic and yolk sac growth in *in vitro* conceptus culture in serum medium containing microsomes. Day 11 rat conceptuses were cultured for 46–48 hr in heat-activated rat serum containing the indicated additions. Data are the mean ± S.E. for five or more conceptuses. Values significantly different from controls are indicated with one ($P < 0.01$) or two ($P < 0.001$) asterisks.

Table 1. Effect of increasing NADPH concentration on cyclophosphamide-induced alterations in conceptus structure and function in the *in vitro* microsomal activating/embryo culture system*

Treatment	Yolk sac diameter (mm)	Somite number in embryos	Embryos with heartbeat	Abnormal embryos
Control	7.46 ± 0.15 (18)†	38.4 ± 0.36 (18)†	18/18	0/18
Cyclophosphamide	7.29 ± 0.23 (7)	38.3 ± 0.42 (7)	7/7	0/7
NADPH (1.0 mM), microsomes	7.38 ± 0.15 (8)	38.8 ± 0.31 (8)	8/8	0/8
NADPH (0.2 mM), cyclophosphamide, microsomes	6.76 ± 0.15‡ (5)	34.3 ± 0.98§ (5)	5/5	4/5§
NADPH (0.5 mM), cyclophosphamide, microsomes	6.03 ± 0.17§ (6)	27.2 ± 1.24§ (5)	2/5‖	6/6§
NADPH (1.0 mM), cyclophosphamide, microsomes	5.21 ± 0.24§ (11)	20.0 ± 1.41§ (5) 6 not countable	1/11§	11/11§

* Day 11 rat conceptuses were cultured for 46–48 hr in heat-activated rat serum with the additions indicated. The concentrations of cyclophosphamide and microsomal protein used were 0.35 mM and 0.4 to 0.5 mg/ml respectively.

† Values are means ± S.E.M. (N).

‡ $P < 0.05$, when compared with controls.

§ $P < 0.001$, when compared with controls.

‖ $P < 0.01$, when compared with controls.

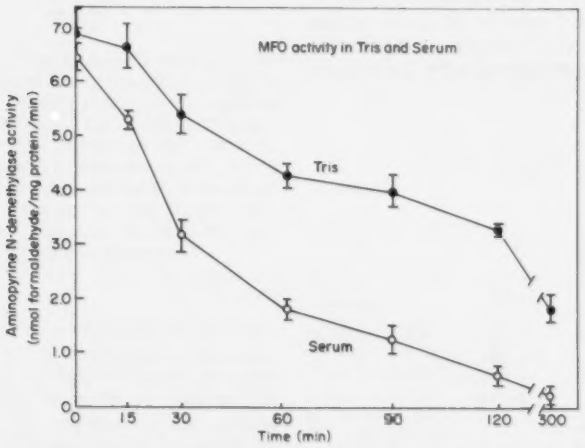

Fig. 2. Time course study of microsomal aminopyrine *N*-demethylase activity in 0.05 M Tris–HCl buffer, pH 7.5, and heat-inactivated rat serum. The gas phase used in conceptus culture (5% CO_2, 20% O_2, and 75% N_2) was used with the serum study; air was used for the enzyme activity measurements done in Tris–HCl buffer

system, however, cyclophosphamide produced significant decreases in final yolk sac and embryonic DNA and protein content. In order to determine if these effects were cytochrome P-450 dependent, the concentration of NADPH was increased from 0.2 to 0.5 and 1.0 mM (Fig. 1). Increasingly higher concentrations of NADPH in the serum medium containing the same levels of cyclophosphamide and microsomes produced dose-related decreases in embryonic and yolk sac growth. For example, embryonic DNA contents were 73, 37 and 17 per cent of control levels after incubation with cyclophosphamide, microsomes, and 0.2, 0.5 or 1.0 mM NADPH respectively.

There were morphological and functional changes in the embryos exposed to cyclophosphamide in the coupled microsomal activating/embryo culture system. Although no grossly abnormal embryos were noted in eighteen controls, twenty-one of twenty-two embryos exposed to NADPH, microsomes, and cyclophosphamide exhibited morphological lesions. Cyclophosphamide produced NADPH-dependent

decreases in yolk sac diameter, embryo somite number, heart function, and a heightened incidence of abnormal embryos (Table 1).

Although microsomes in serum were initially almost as metabolically active in *N*-demethylating aminopyrine as they were in Tris–HCl buffer, it seemed possible that the metabolic activity of microsomes in serum would not last as long as it did in Tris–HCl buffer. For that reason, a time course comparison of the aminopyrine *N*-demethylase activities during the first 2 hr of incubation at 37° was performed (Fig. 2). The enzymatic ability of microsomes to *N*-demethylate aminopyrine fell much more rapidly in serum than in Tris buffer, and by 120 min after the start of incubation, only 10 per cent of the initial activities remained.

To ascertain the importance of metabolism in these NADPH-dependent cyclophosphamide-induced decreases in conceptus growth, an experiment was performed to differentiate between mechanisms of cyclophosphamide metabolism by microsomes and mechanisms involving a damaged yolk sac. For example, the presence of microsomes and NADPH injures the yolk sac [22] and, thus, might allow transport of more unmetabolized cyclophosphamide, producing the toxic effect observed. Therefore, day 11 rat conceptuses were incubated for 2 hr in medium containing 1.0 mM NADPH and microsomes. The conceptuses were then removed from the medium, washed three times in Trowell's T8 tissue culture medium, and then placed in fresh rat serum containing 0.35 mM cyclophosphamide for the remaining 46 hr of culture time. The growth of these conceptuses was not significantly different from that of similarly handled controls and was significantly higher than values obtained when NADPH, microsomes, and cyclophosphamide were present together for the first 2 hr of incubation (Fig. 3). Even though the microsomal aminopyrine *N*-demethylase activity was diminished by 90 per cent 2 hr after the incubation was started, the toxic effect of the combined treatment group of microsomes, NADPH, and cyclophosphamide was not fully initiated at the 2-hr time point. Conceptuses cultured with these three agents for 48 hr showed a larger toxic effect.

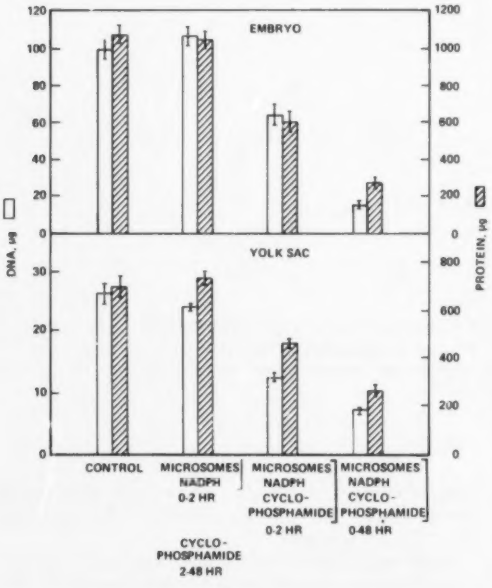

Fig. 3. Effects of time of incubation and of chemical treatment grouping on the cyclophosphamide-induced decreases in embryo and yolk sac growth. Day 11 rat conceptuses were cultured, with the additions indicated, in rat serum for the specified times as described in Methods. Data are the mean ± S.E. for five or more determinations.

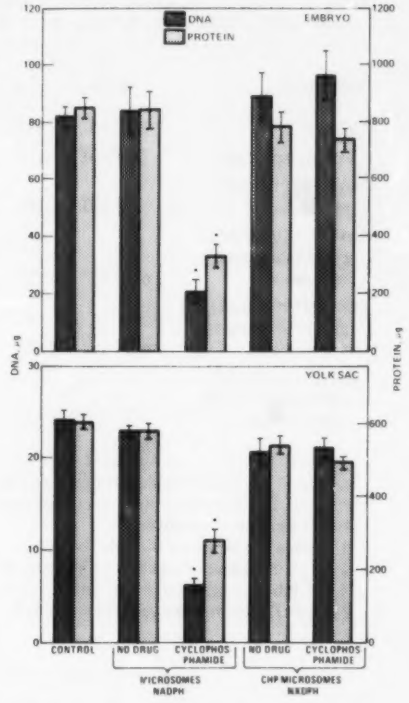

Fig. 4. Effects of control and of cumene hydroperoxide (CHP)-treated microsomes of cyclophosphamide-induced decreases in conceptus growth. Day 11 rat conceptuses were cultured with 0.5 mM NADPH, cyclophosphamide (0.35 mM), and microsomes (0.21 mg protein/ml for control and 0.17 mg protein/ml for cumene hydroperoxide-treated) for 46–48 hr. Final DNA and protein levels in the embryo and yolk sac are the mean ± S.E.M. for five or more determinations. An asterisk (*) indicates $P < 0.001$ when compared with the no drug control.

To further implicate the role of cytochrome P-450 in the activation of cyclophosphamide to metabolites that are toxic and teratogenic to embryos cultured *in vitro*, cumene hydroperoxide-pretreated, and thus cytochrome P-450-depleted, microsomes were used. Figure 4 compares the effects of cyclophosphamide in coupled microsomal activating/embryo culture systems containing normal or cytochrome P-450-deficient microsomes. Rat conceptuses cultured in medium containing either microsomes and NADPH or cumene hydroperoxide-treated microsomes and

NADPH did not grow significantly less than controls. However, when cyclophosphamide, microsomes, and NADPH were all present in the serum medium together, significant decreases in embryonic and yolk sac growth were observed. In this combined treatment group, the yolk sac DNA and protein values were only 27 and 50 per cent of controls, whereas the embryonic DNA and protein contents were 26 and 37 per cent of normal values. When rat conceptuses were cultured in serum media containing cyclophosphamide, cumene hydroperoxide-treated microsomes and NADPH, however, no significant decreases in growth were observed compared to the cumene hydroperoxide-treated microsomes and NADPH treatment group.

The functional and structural assessment of rat embryos in the five treatment groups of Table 2 agrees closely with the overall embryo and yolk sac growth data. The presence of cyclophosphamide in serum medium containing NADPH and microsomes caused significant decreases in yolk sac diameter, embryo somite number, and heart beat incidence, and increased the incidence of structural embryo abnormalities. In contrast, serum medium containing cyclophosphamide, NADPH, and cumene hydroperoxide-treated microsomes did not significantly lower the yolk sac diameter, the embryo somite number, or the incidence of a beating heart, nor did it cause structural abnormalities to develop in the differentiating rat embryos.

DISCUSSION

Studies in the coupled microsomal activating/embryo culture system indicate that metabolism of cyclophosphamide appears to be necessary to produce toxic and teratogenic effects *in vitro*. Cyclophosphamide had no deleterous effects on conceptus growth or differentiation in the absence of microsomes and NADPH. The toxicity and teratogenicity of cyclophosphamide observed in the coupled microsomal activating/embryo culture system was NADPH-dependent, indicating that an NADPH-dependent route of metabolism, such as cytochrome P-450, is a requirement for the bioactivation of cyclophosphamide to toxic and teratogenic metabolites. Experiments in which microsomes and NADPH were present in the conceptus incubation medium for the first 2 hr followed by culturing the washed conceptuses in fresh medium containing cyclophosphamide, showed no decrease in embryo growth or altered differentiation. Metabolism, rather than a mechanism involving a damaged yolk sac, is the most reasonable cause of the effect of the microsomal activating system on the conceptus culture system. If microsomes and NADPH-induced changes in the yolk sac increased transport of cyclophosphamide into the conceptus, and unmetabolized cyclophosphamide were embryotoxic and teratogenic, it would be expected that this treatment group would have shown adverse effects. It did not. Combining cyclophosphamide, microsomes, and NADPH into one treatment group for the initial 2 hr did not produce as large an effect as a full 48 hr exposure. Recently, 4-hydroxycyclophosphamide/aldophosphamide has been detected in the serum of cyclophosphamide-treated mice and proposed as a transport form of the ultimate toxic metabolites of cyclophosphamide [23]. The third argument implicating cytochrome P-450-dependent metabolism of cyclophosphamide involves the differences observed when untreated hepatic microsomes or microsomes partially depleted of cytochrome P-450 were used in the coupled microsomal activating/embryo culture system. Cumene hydroperoxide-pretreated hepatic microsomes used in these studies exhibited only 24 per cent of control

Table 2. Effect of untreated and cumene hydroperoxide-treated microsomes on cyclophosphamide-induced alterations in embryo structure and function*

Treatment	Yolk sac diameter (mm)	Somite number in embryos	Embryos with heartbeat	Abnormal embryos
Control	7.38 ± 0.08 (8)†	38.1 ± 0.40 (8)†	8/8	0/8
Microsomes, NADPH	7.18 ± 0.11 (8)	37.6 ± 0.32 (8)	8/8	0/8
Microsomes, NADPH, cyclophosphamide	6.02 ± 0.16‡ (8)	26.5 ± 0.89‡ (6)	3/8§	8/8‡
Cumene hydroperoxide-treated microsomes, NADPH	7.01 ± 0.11 (8)	37.8 ± 0.53 (8)	8/8	0/8
Cumene hydroperoxide-treated microsomes, NADPH, cyclophosphamide	6.76 ± 0.11 (8)	38.4 ± 0.38 (8)	8/8	0/8

* Day 11 rat conceptuses were cultured for 46–48 hr in heat-inactivated rat serum with the indicated additions. NADPH and cyclophosphamide concentrations were 0.5 mM and 0.35 mM respectively. Statistical comparisons have been made between the no drug and cyclophosphamide included treatment for the untreated microsomal and cumene hydroperoxide-treated microsomal groups.

† Values are means ± S.E.M. (N).

‡ $P < 0.001$.

§ $P < 0.05$.

cytochrome P-450 levels and only 15 per cent of control aminopyrine *N*-demethylase activities. Previous work from our laboratory demonstrated that *in vitro* cumene hydroperoxide treatment of hepatic microsomes from male rats reduced the cytochrome P-450 content and the benzphetamine *N*-demethylase and aryl hydrocarbon hydroxylase activities to only 20, 10 and 2 per cent of control values respectively [24]. Including these cumene hydroperoxide-treated microsomes with NADPH and cyclophosphamide with conceptuses cultured *in vitro* showed reduced growth inhibition when compared to untreated microsomes.

The results of this study on the requirement for microsomes and NADPH to bioactivate cyclophosphamide to metabolites that are toxic and teratogenic to cultured conceptuses are essentially in agreement with the study of Fantel *et al.* [9]. They showed that both decreased embryonic growth and increased embryonic abnormalities correlated with increasing cyclophosphamide concentrations in human serum medium containing a hepatic post-mitochondrial supernatant fraction from Aroclor 1254-pretreated rats. In the studies presented here, we observed morphological effects such as blunted tails, stunted limb buds, and mandibular arch irregularities which agree closely with the study of Fantel *et al.* [9]. Additionally, we observed olfactory, otic, and optic abnormalities and stunted or bifurcated snouts in embryos grown in medium containing cyclophosphamide, microsomes, and various amounts of NADPH. In our studies, yolk sac circulation was also adversely affected in the coupled system with 1.0 mM NADPH, microsomes, and cyclophosphamide.

Comparison of *in vitro* embryo culture *per se* with embryo culture in medium containing a microsomal activating system can help define the role of the maternal drug metabolism in embryo toxicity and teratogenicity. Additionally, the coupled microsomal activating/embryo culture system may be used as an embryo toxicity and teratogenicity test or screen. Kao *et al.* [25] have shown that with $CdCl_2$ and valproate, two teratogens of diverse chemical structure, similar morphological lesions are induced in post-implantation embryo culture and *in vivo*. Only small quantities of chemicals are needed to examine their effects on this *in vitro* system as compared to the larger mass required for *in vivo* animal tests. For example, only 500 μg of compound is necessary to treat ten separate embryos (which could be obtained from ten separate dams for statistical purposes) with 10 μg/ml of the test chemical. With 2,4,6-triethylenimino-1,3,5-triazine, as little as 0.05 μg/ml produces toxic and teratogenic effects in *in vitro* embryo culture [26]. Thus, experiments with the coupled microsomal activating/embryo culture will be useful for studying *in vitro* mechanisms of anomalous development, particularly the role of maternal metabolism in embryo toxicity and teratogenicity, as well as providing a possible Ames-like embryonic toxicity and teratogenicity test system.

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